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Communication networks in the zebrafish, *Danio rerio*

Ana Sofia Carvalho Cruz

Dissertação

Mestrado de Biologia Evolutiva e do Desenvolvimento

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RESUMO

Muitos comportamentos sociais dependem de eventos de comunicação entre, pelo menos, dois indivíduos (McGregor & Peake, 2000). Estes emissores e recetores recíprocos de sinais trocam informação potencialmente relevante entre si. Contudo, a comunicação não está somente restrita a estas interações diádicas. De facto, os sinais trocados entre dois animais podem estar disponíveis a um grande número de outros indivíduos, ao mesmo tempo. Assim, o ambiente social é composto por uma extensa rede de comunicação, composta não só por sinalizadores e recetores mas também por indivíduos capazes de intercetar a informação trocada entre outros (McGregor & Peake, 2000). Esta forma de aprendizagem social em que terceiros adquirem informação através da interceção de sinais e usam-na posteriormente é denominada de *Eavesdropping* (McGregor, 1993).

Adquirir informação através de aprendizagem social pode ser benéfica para os intercetores. De facto, os indivíduos podem reunir informação sobre os seus conspecificos sem os custos associados à aprendizagem de tentativa-e-erro (Danchin, Giraldeau, Valone, & Wagner, 2004). Por exemplo, um macho pode informar-se acerca da capacidade combativa de outro sem a ocorrência de confronto direto, diminuindo assim o risco de dano ou morte e, ao mesmo tempo, atualizando a sua perceção do rival (McGregor, 1993). Este tipo de aquisição de informação tem sido maioritariamente estudada em dois contextos: (1) contexto da escolha de parceiro; (2) contexto de disputa agressiva (Valone, 2007).

Na procura por um parceiro, as fêmeas poderão adquirir informação acerca de qualidade de futuros parceiros através de *eavesdropping,* nomeadamente através da observação ou escuta de interações entre machos. Este fenómeno tem sido reportado em várias espécies, incluíndo aves (Amy et al., 2008; Garcia-Fernandez, Amy, Lacroix, Malacarne, & Leboucher, 2010; Ophir & Galef, 2003; Otter et al., 1999) e peixes (Claire Doutrelant & McGregor, 2000).

Para além da troca de sinais entre machos, as fêmeas podem interceptar a troca de sinais feita em interações machos-fêmea (Valone, 2007). Vários estudos referem-se a este fenómeno como *mate choice copying* (ou cópia da escolha de parceiro) uma vez

que, na maioria das vezes, as fêmeas que intercetam sinais destas interações copiam a escolha da fêmea observada (Danchin et al., 2004). Este fenómeno tem sido extensivamente estudado, em várias espécies (Alonzo, 2008; Amlacher & Dugatkin, 2005; Dugatkin & Godin, 1992; Galef & White, 1998; Swaddle, Cathey, Correll, & Hodkinson, 2005; Witte & Ryan, 2002). Nestes estudos, as fêmeas revertem a sua preferência inicial em relação a um potencial parceiro, após observarem ou escutarem um macho não preferido a interagir com outra fêmea.

A aquisição de informação através da interceção de sinais por terceiros também pode ser valiosa para machos, presentes num contexto agressivo. De facto, os machos podem intercetar sinais provenientes de interações entre outros machos de forma a adquirirem informação sobre a capacidade combativa destes (Valone, 2007) e, consequentemente, adaptarem o seu comportamento face a cada um deles. Novamente, os custos de aprendizagem tipo tentativa-e-erro são diminuídos. Discriminação na resposta face a vencedores e perdedores de interações intercetadas foi já detetada em várias espécies, nomeadamente aves, peixes e mamíferos (Amy & Leboucher, 2009; Grosenick, Clement, & Fernald, 2007; Johnsson & Åkerman, 1998; Lai, Yu, Liu, Kuo, & Huang, 2014; Naguib, Fichtel, & Todt, 1999; Oliveira, McGregor, & Latruffe, 1998; Peake, Terry, McGregor, & Dabelsteen, 2001).

Apesar da aquisição de informação através deste tipo de aprendizagem social conferir vantagens óbvias, os animais também recolhem informação importante através das suas interações próprias com o ambiente social (Dall, Giraldeau, Olsson, McNamara, & Stephens, 2005). Como tal, é expectável que uma integração entre a de informação adquirida através da experiência própria e de *eavesdropping* ocorra. De facto, experienciar uma vitória ou derrota após uma interação agressiva pode influenciar fortemente o comportamento agonístico de um indivíduo *à posteriori* (Rutte, Taborsky, & Brinkhof, 2006). Por exemplo, no hamster-sírio (*Mesocricetus auratus),* uma experienciar uma derrota influencia o uso de informação adquirida através da observação de uma interação entre dois machos (Lai et al., 2014).

A presença de terceiros, capazes de detetar e intercetar sinais pode promover a emergência de uma situação em que, a presença destes indivíduos pode potencialmente

influenciar o comportamento sinalizador de outros. Este fenómeno é denominado por efeito de audiência (Marler, Dufty, & Pickert, 1986) e tem sido reportado em vários estudos (Baltz & Clark, 1997; Bertucci, Matos, & Dabelsteen, 2013; Dzieweczynski, Greaney, & Mannion, 2014; Dzieweczynski & Perazio, 2012; Leaver, Hopewell, Caldwell, & Mallarky, 2007;Matos, Peake, & McGregor, 2003; Plath, Blum, Schlupp, & Tiedemann, 2008). O efeito de uma audiência tem sido maioritariamente estudado, tal como o *eavesdropping,* no contexto agonístico. Por exemplo, machos de grilo-do-campo (*Gryllus veletis*) são mais agressivos numa interação com outro macho, quando uma audiência está presente, seja esta composta por machos ou fêmeas. Contudo, o número de exibições de vitória, após vencerem um encontro agonístico, aumentou quando uma audiência masculina está presente(Fitzsimmons & Bertram, 2013).

O objetivo deste trabalho foi determinar a existência destes dois fenómenos associados à existência de redes de comunicação, o *eavesdropping* e os efeitos de audiência, em peixe zebra (*Danio rerio*). Esta espécie, para além de viver num contexto social e demonstrar comportamento agressivo territorial (Oliveira, Silva, & Simões, 2011; Spence, Gerlach, Lawrence, & Smith, 2008), é um organismo modelo usado em áreas como o estudo do desenvolvimento, ecologia ou neurociências (Oliveira, 2013). Sendo uma espécie bem caraterizada, o seu uso permite uma abordagem mais completa a estudos comportamentais devido à existência de variadas ferramentas genéticas, como linhagens transgénicas ou mutantes (Oliveira, 2013).

Numa primeira experiência testámos a existência de *eavesdropping* em contexto agressivo. Para tal, machos de peixe-zebra foram expostos uma interação entre dois demonstradores e posteriormente, a sua preferência em relação a cada um deles (vencedores e perdedores) foi analisada, através da contabilização do tempo passado perto de cada um. Também testámos qual o efeito de experienciar uma vitória ou uma derrota no uso da informação recolhida pela observação dos demonstradores, ao submeter os futuros observadores a interações agressivas entre si. Nesta experiência, nenhuma preferência em relação aos vencedores ou perdedores foi detetada. Devido à obtenção deste resultado, também não foi possível determinar a influência da experiência passada, uma vitória ou uma derrota, no uso da informação adquirida através da observação de interações agonísticas.

Numa segunda experiência, tentámos determinar se a presença visual de uma audiência mista de conspecificos durante uma interação entre dois machos promoveria uma alteração do seu comportamento agressivo. Estávamos também interessados em determinar se a pré-exposição a uma audiência antes da interação agonística decorrer, poderia influenciar o comportamento agressivo destes macho - *priming* agressivo (Matos et al., 2003). Para tal medimos vários comportamentos agonísticos entre pares de machos, nomeadamente comportamento de agressão indireta, como por exemplo exibições, ou direta, como mordidas ou perseguições (Oliveira et al., 2011). Detetámos um aumento da agressão indireta quando uma audiência de conspecificos está presente durante a interação. Contudo, a pré-exposição a conspecificos promove o aumento do comportamento de perseguição.

Neste trabalho demonstrámos a existência de efeitos de audiência em peixe-zebra, embora não tenhamos conseguido detetar a ocorrência de *eavesdropping*. Porém, novos estudos deverão ser desenvolvidos de forma a implementar um paradigma comportamental que demonstre a existência de *eavesdropping* nesta espécie. Tal poderá, no futuro, promover uma abordagem mais completa do estudo de redes de comunicação, que inclua não só uma análise comportamental e hormonal mas também uma análise das redes génicas e circuitos neuronais associados a estes fenómenos.

PALAVRAS-CHAVE: Redes de Comunicação; *Eavesdropping;* Efeitos de Audiência; Peixe-zebra; Aprendizagem Social.

ABSTRACT

Communication is not only restricted to dyadic interactions. And, therefore, signals exchanged between animals can be available to third-party elements, that can gather information potentially relevant to them, in a phenomenon termed as eavesdropping. For instance, in an aggressive context, males could eavesdrop on male-male interactions to gather information about their rivals' fighting abilities. However, the presence of bystanders can lead to a situation in which their presence could potentially influence the signaling behaviour of eavesdropped animals, in a phenomenon named as audience effect. The effect of an audience on agonistic signaling behaviour has been extensively studied in several species.

Here, we were interested in determining if zebrafish males (*Danio rerio*) could eavesdrop on aggressive contests between demonstrators and, if so, whether experiencing a prior victory or defeat could influence how they use the gathered information. To do this, we measured their preference in terms of the time spent with each winner or loser. Also, we wanted to determine if males adjusted their agonistic behaviour when exposed to conspecifics, either before and/or during an aggressive interaction. In these experiments we used zebrafish since it has been used as a model organism in several areas of study. Therefore, by being a highly studied species, it has an extensive number of genetic resources available such as transgenic or mutant lines.

We could not detected the presence of eavesdropping in zebrafish in terms of its preference towards winners or losers of seen interactions. Therefore we could not determine the influence of a prior outcome in the use of socially acquired information. However, we found that zebrafish males adjust their aggressive behaviour when conspecifics are present, by increasing the frequency of assessment in these interactions. Also, pre-exposure to conspecifics increased the time winners spent chasing losers.

KEYWORDS: Communication Networks; Eavesdropping; Audience Effects; Zebrafish; Social Learning.

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1.1. ANIMALS COMMUNICATE THROUGH SIGNALS IN COMMUNICATION NETWORKS

Social behavior and communication are interconnected, since many important social behaviors, with a profound impact on an individual's fitness, depend upon the communication between, at least, two individuals (McGregor & Peake, 2000). For instance, communication between animals is crucial in a mating or territorial context (McGregor, 1993).

Animals are then reciprocal signalers and receivers that interchange signals, between one another. A signal is a feature or a behavior that has been shaped by natural selection and that provides information to an intended receiver. Signals should then evoke from this last one a beneficial response to the signaler (Goodale, Beauchamp, Magrath, Nieh, & Ruxton, 2010), being widely used in communication events between conspecifics. Alarm calls or mating vocalizations are examples of signals relevant to ones' fitness (Valone, 2007), since they increase the signaler's chances of survival or reproductive success.

Although signals allow the flow of potentially relevant information between signalers and receivers, communication is not only restricted to dyadic interactions. In fact, the social environment is composed by many animals, which can simultaneously act as signalers or receivers. And, because animals communicate, sometimes at great distance, conspicuous signals sent between them can be available to several individuals at the same time. Thus, the social environment comprises large communication networks, composed not only by alternate signalers or receivers but also peripheral individuals, capable of detecting the information flow between others (McGregor & Peake, 2000).

1.2. EAVESDROPPING: USING OTHERS' SIGNALS THROUGH SOCIAL LEARNING

As so, when included in communication networks, animals have an opportunity to acquire information through the interception of signals, as third-party elements. This phenomenon has been termed eavesdropping (McGregor, 1993).

It is easy to understand how acquiring social information through eavesdropping can be benefitial to bystanding animals. When perceiving interactions between third-parties, bystanders can effectivelly gather information about observed conspecifics without the costs associated with trial-and-error tactics (Danchin et al., 2004), therefore being an effective way of learning about their social environment. For instance, a male can inform himself about another male's fighting ability without directly confronting it, hence, decreasing the risk of injury or death while, at the same time, updating its information about a potential rival (McGregor, 1993). This type of information acquisition has mainly been studied in two different contexts: mating decisions and agressive disputes (Valone, 2007).

A) USING SIGNALS TO ASSESS MATE QUALITY

When searching for a mate, females usually prefer high quality males and can often use the direct signals sent by them to acess their quality. For instance in the three-spined stickleback (*Gasterosteus aculeatus*), non-parasitized males exhibit a red colored belly, which is more atractive to females (Milinski & Bakker, 1990). However, eavesdropping on potential mates' interactions can also provide reliable information about their quality. Indeed, females can eavesdop on male-male contests to assess the quality of their potential mates.

In a study with fighting fish (*Betta splendens*) (Doutrelant & McGregor, 2000), females observed a 15 minute interaction between two males and then were allowed to visit them. After observing the contest, females spent more time with the winner, also displaying a reproductive color towards it. Females that were not allowed to observe the interaction did not behave differently towards the winner of the unseen interaction.

Because a 15 minute interaction is not enough to incite changes on the coloration of loser males, these results suggest that females actually eavesdropped on male-male contests. Similar results were found in great tits (*Parus major*), in which females assess male quality by eavesdropping on song contest outcomes, invading the territories of neighboring males when their own mate's song is overlapped by a playback song (Otter et al., 1999)

However, females may also prefer losers over winners after eavesdropping on male-male interactions. This is the case of the Japanese quail (*Coturnix japonica*), in which winners are more aggressive towards females when courting them (Ophir & Galef, 2003).

Female canaries (*Serinus canaria*) can also eavesdrop on their future mates, whether it is through song contests or physical competitions for food (Amy et al., 2008). In both cases, females used the outcome of these interactions to direct their behavior towards males. After earring a simulated song contest between two speakers, they performed more displays when the overlapping song was played. However, when females observed physical interactions between males, they spent more time with the losers of such contests. As so, not only females use eavesdropping to gather information about potential partners, but also behave differently according to the type of signal by which they acquire information (Amy et al., 2008). Song contests between male canaries may also influence female maternal investment. Females of this species were exposed to a fabricated song interaction in which one song overlapped the other. After five days, females were either exposed to the overlapping or overlapped song twice a day until egg laying ended. It was found that females exposed to the overlapping song increased the yolk content of their eggs (Garcia-Fernandez et al., 2010).

Although it is true that females eavesdrop on male-male interactions, and that such behavior can impact their perception about their potential or actual mates, they have another source of information in hand. Indeed, eavesdropping on male-female interactions can also influence female mate choice (Valone, 2007). Several studies have referred to this phenomenon as mate choice copying, since most of the times, after observing a male-female interaction between nearby conspecifics, bystanders copy the

eavesdropped female's choice (Danchin et al., 2004). This source of information is used more often by inexperienced younger females that copy the mating decisions of older, more experienced females (Amlacher & Dugatkin, 2005), especially if they cannot distinguish between high and low quality males (Valone, 2007).

In zebra finches, females showed a preference for males with a female partner, rather than males that share their cage with other males (Swaddle et al., 2005). Also, in the Japanese quail, females reversed their mate preference after observing the non-preferred male interacting with another female (Galef & White, 1998) . Similar results were found in the guppy (*Poecilia reticulata*), as females' innate preferences were reversed after observing females spending time with their less preferred males (Dugatkin & Godin, 1992).

Some studies have now addressed mate choice copying in the wild. Actually, it was shown that ocellated wrasse (*Symphodus ocellatus*) females copy the mating decisions of others in their natural environment (Alonzo, 2008). This same result was obtained in the Sailfin molly, *Poecilia latipinna* (Witte & Ryan, 2002), where females visit males more often when another female is nearby. These studies performed in natural conditions replicate the results obtained in laboratory which might indicate that mate choice copying can also occur in nature. And, if so, eavesdropping might be having a profound impact on the females' mating decisions or extra-pair behavior.

B) USING SIGNALS TO ACESS A RIVAL'S AGONISTIC ABILITIES

Females eavesdrop on males to access their quality, but eavesdropping can also be valuable in an aggressive territorial context. In fact, males can eavesdrop on aggressive interactions between neighboring males to acquire information about their rivals' fighting abilities (Valone, 2007), thus avoiding costly interactions by adapting their behavior towards them. For instance, like female fighting fish, males eavesdrop on agonistic interactions between rivals. After observing a fight, and therefore its outcome, males responded differently to a territorial intrusion performed by the winner or loser of the observed interaction. Indeed, bystanders took more time to approach and display to

seen winners than to seen losers. However, no such difference was found when faced with unseen winners or losers (Oliveira, McGregor, & Latruffe, 1998).

Male great tits also gather information by eavesdropping on vocal interactions between competitors (Peake, Terry, McGregor, & Dabelsteen, 2001). In this study, experimenters simulated an interaction between two males using speakers and song overlapping as a signal of willingness to escalate the confrontation. After earring a vocal confrontation outside their territory and then perceiving a simulated territorial intrusion by the overlapped male, bystanders decreased the number of songs produced. A similar study was performed in nightingales (*Luscinia megarhynchos*) (Naguib et al., 1999). After listening to a simulated interaction between two males the bystanders' response to an overlapper male was stronger. Indeed, they spent more time singing to the overlappers, used more songs and the latency to approach them was lower. Even when the overlapper male was silent and the overlapped male was not, bystanders still tended to approach and sang more towards the overlapper (Naguib et al., 1999) . Green swordtails (*Xiphophorus helleri*) also modulate their aggressive behavior towards winners and losers of previously seen interactions (Earley & Dugatkin, 2002), as they are less likely to initiate an interaction with the seen winners. Also, their probability of winning such contest is significantly decreased. Discrimination in the response towards losers or winners of seen interactions was also found in the rainbow trout (*Oncorhynchus mykiss*) (Johnsson & Åkerman, 1998), male canaries (Amy & Leboucher, 2009), and golden hamsters (*Mesocricetus auratus)* (Lai et al 2014).

Eavesdropping on disputes between males has been also linked to a more complex cognitive ability - transitive inference. Through observation of a series of interactions between competitors, males could infer dominance hierarchies between them. In a study made with tilapia (*Astotilapia burtoni*), bystanders observed several sequential disputes between nearby rivals. Afterwards, their preference for males positioned in different ranks of the dominance hierarchy was tested. And, even though the bystanders never saw these males directly contact with one another, they spent more time with the low ranked male (Grosenick, Clement, & Fernald, 2007). Similar results were obtained in hens (*Gallus gallus domesticus*) (Hogue, Beaugrand, & Laguë, 1996). After observing a

stranger male winning a known dominant male, bystanders were more likely to lose an interaction with the unknown male.

C) DOMINANCE STATUS AND THE USE OF EAVESDROPPED INFORMATION

Several studies have demonstrated that animals can use information obtained by eavesdropping, both on a territorial or mating context. However, animals can also gather information through their personal experience in the social environment (Dall et al., 2005) and, as so, one might expect that such interactions could potentially affect the use of eavesdropped information. For instance, it is known that victories and defeats can have an impact on an individuals' subsequent behavior (Rutte et al., 2006). In zebrafish males, winning or losing a contest influences the outcome of a second encounter with another competitor. After winning a dispute, zebrafish males increased their chances of winning a second dispute with a naive individual. On the other hand, losing disputes increased the probability of losing a posterior contest (Oliveira, Silva, & Simões, 2011). These so called winner and loser effects, respectively, have been detected in several species. For instance, in male crickets (*Gryllus bimaculatus*), prior outcomes can influence the dominance relations between them. In dominance hierarchies, second ranked males lost their dominance towards third ranked males after experiencing a defeat. On the opposite side, third rank males won an encounter with a dominant male after winning a previous contest (Khazraïe & Campan, 1999). Also, in spiders (*Argyrodes antipodiana*), trained winners are more likely to win contests with trained losers (Whitehouse, 1997).

These past fighting experiences might influence how animals use gathered information about other males. In the golden hamster, experiencing a defeat affected use of eavesdropped information. Animals with no previous fighting experience, spent more time near the winner of a seen interaction between demonstrators. However, when bystanders experienced a defeat before observing this contest, they spent less time with the seen winner *(*Lai et al*.*, 2014). This result might indicate that losing a previous contest can influence how animals respond to winners or losers of seen interactions.

1.3. AUDIENCE EFFECTS : MANIPULATING SIGNALS IN THE PRESENCE OF EAVESDROPPERS

As we have seen, the social environment comprises signalers, receivers and also potential eavesdroppers. It is therefore expected that the presence of such individuals could potentially affect the behavior of signalers. These so called audience effects refer to a situation in which eavesdropped animals manipulate their signaling behavior in the presence of unintended viewers (Marler et al., 1986). As so, these bystanders are not only influenced by the signals they eavesdrop on but are simultaneously influencing the interactions between other animals (Earley & Dugatkin, 2002). Because of this, signalers can manipulate their signals' conspicuousness or intensity in the presence of bystanders if that is beneficial to them. For instance, when the presence of potential eavesdroppers is detrimental, signalers can restrict the spread of their own signals (McGregor & Peake, 2000). In the European grey squirrel (*Sciurus carolinensis*), the presence of conspecifics during foraging and caching increases the risk of food pilferage. For this reason, grey squirrels increase the distance between caches and orient themselves by facing away from the audience when foraging (Leaver et al., 2007). On the opposite situation, signalers might enhance their signals when bystanders are around. Indeed, in fiddler crabs (*Uca annulipes),* females prefer males that wave their enlarged claw faster (Backwell, Jennions, Christy & Passmore, 1999). When a male has an opportunity to mate, and an audience of other competitors is in sight, they increase their claw's wave rate (Milner, Jennions, & Backwell, 2012)

Audience effects seem to be a wide-spread phenomenon as numerous studies have detected the impact of the presence of eavesdroppers on the signalers' behaviour. For example, the yellow mongoose (*Cynictis penicillata*) changes its alarm calling behavior in the presence of conspecifics. After detecting a predator, these individuals will only use a vocal alarm call when they are accompanied by others (le Roux, Cherry, & Manser, 2008).

The presence of conspecifics also influences the mating choices of observed individuals. This is the case of the Atlantic mollies (*Poecilia mexicana*), a species in which males

choose their mates, preferring larger over smaller females. However, when a male competitor was present, this preference was lost and subjects spent the same amount of time with both females (Plath & Schlupp, 2008). As so males mollies hidden their innate preference by larger females, when another competitor is nearby, since it could potentially copy its mate choice, therefore decreasing the observed male reproductive fitness (Ziege et al., 2009). Similar results were found in a study made with cave mollies, the cave dwelling form of *Poecilia mexicana*. Despite their smaller eyes, the visual presence of another male promotes a change on the subjects' innate preferences - that is, males spend less time with their preferred female (Blum, Plath, Tiedemann, & Schlupp, 2008).

Audience effects not only depend upon the presence of conspecifics. Other factors such as sex composition of the audience or familiarity between signalers and bystanders can have an impact on the modulation of their signaling behavior. For instance, in fighting fish, males decreased their aggressiveness towards an opponent when facing a female audience, rather than a male audience. They diminish the number of bites and the time spent facing the opponent, also increasing behavioural displays used both in aggression and courtship (Doutrelant, McGregor, & Oliveira, 2001). The fact that highly agressive males can drive away females might explain this adjustment of aggressiveness (Matos & McGregor, 2002). As so, the modulation agressive signaling can potentially increase the observed male's reproductive fitness. The effect of a same-sex audience has also been recently found between interacting females of this species (Dzieweczynski, Greaney, & Mannion, 2014). Indeed, when interacting with another female, focal subjects adjust their signaling behavior towards it when a bystander is present. The time spent gill flaring or performing opponent-directed tracking, as well as the number of tail beats increased when a conspecific was around, especially if this bystander was a female.

The same type of signaling adjustment, according to the audience's sex composition, was also studied in an invertebrate. The male field cricket was more aggressive when an audience, either composed by a male or female, is present. However, the frequency of victory displays from the winner increased only when a male audience was present (Fitzsimmons & Bertram, 2013).

Familiarity between subjects and the audience elements is another factor to account for, since it can also influence male-male interactions (Dzieweczynski & Perazio, 2012). In Siamese fighting fish males, the presence of unfamiliar male audiences, promoted an increase of the time subjects spent displaying towards both the opponent and bystander. They also spent more time near the audience tank. This results suggests that prior social exposure to males in an the audience can influence agressive signaling of observed males.

In this species, the time of presentation of an audience can also affect the signaler's behaviour. That is, pre-exposing an individual to an audience can influence the way it behaves afterwards. When male subjects were pre-exposed to the audience they decreased the latency until the first bite event (Matos, Peake, & McGregor, 2003). As so, although the bystander was not present at the time of the conflict, subjects still adjusted their signaling behaviour, by increasing their aggressiveness towards the opponent - (Priming effect, Matos et al., 2003).

It is important to state that, although less studied, an interspecific audience can also influence the signaling behavior of other animals (Ridley, Child, & Bell, 2007).

1.4. THE INFLUENCE OF EAVESDROPPING AND AUDIENCE EFFECTS ON SOCIAL BEHAVIOUR

The social component of an animal's environment is one of the most unpredictable, since it comprises several behavioural agents interacting with one another (Oliveira, 2012). Thus, gathering information about such an unstable setting can be highly valuable since it is an effective way of evaluating rapid changes occurring around them. In such context, eavesdropping and audience effects can be important tools to navigate these fluctuating environments. On one hand, animals can gather relevant information to direct their behaviors towards others, such as mates or rivals. On the other hand, social behaviour can be modulated in the presence of conspecifics. Animals can then increase their chances of survival or reproductive success either by exploiting or manipulating

signal exchanges occurring in their social environment - a complex and dynamic communication network in which information flows through several signalers and receivers.

1.5. THE USE OF ZEBRAFISH AS A MODEL TO STUDY SOCIAL COGNITION

The existence of audience and eavesdropping effects demonstrate that social context can modulate the animals' behaviour and thus be important to their fitness. Exploring the genetic networks and neural circuits involved in social behaviour modulation is becoming essential to understand how animals acquire information, process it and produce an adequate response towards the social stimuli. To do so, researchers should use animal models with: (1) stereotyped social behavior; (2) a "genetic tool box" which allows to study brain function (Oliveira, 2013).

Zebrafish (*Danio rerio*) is a species which meets both these criteria. Firstly, zebrafish are highly social animals. They live in mixed-sex groups, shoals, can establish dominance hierarchies and demonstrate territorial behaviour (Spence et al., 2008). Indeed, their aggressive behaviour in contests has also been extensively described (Oliveira et al., 2011). Secondly, zebrafish is a model organism used in many different areas such as developmental biology, ecology and neuroscience (Oliveira, 2013). It is a small fish with high reproductive rates (Spence et al., 2008), therefore being easy to accommodate. Their embryos and larvae are transparent which enables a detailed study of their development, and their genetic homology to humans is high (Stewart, Braubach, Spitsbergen, Gerlai, & Kalueff, 2014). Thus, by being highly characterized, it has an impressive *tool box* which includes a whole sequenced genome, detailed brain atlases or GAL-UAS transgenic systems (Oliveira, 2013). Because of these advantages zebrafish is now being considered a good model species to study social cognition.

In fact, a recent study has used zebrafish to investigate how males responds to contest between rivals, by measuring a bystander's attentiveness towards it (Abril de Abreu, et al. unpublished data). In this study, attentiveness was measured through the calculation of bystanders' directionality in its tank as well as the time they spent near

demonstrators' aquarium when aggressive interactions took place. It was actually shown that males spent more time near the demonstrators' aquarium, also spending more time facing them. Such results were not obtained when demonstrator are separated, that is, when the social stimulus is present but no interaction occurs.

2. Paper A

Eavesdropping and the influence of prior fighting experience in zebrafish (*D. rerio*)

In social species, signalers and receivers communicate in complex and dynamic communication network (McGregor & Peake, 2000). In such environment, signal exchange can be intercepted by other animals that might not be directly involved in these interactions (McGregor & Peake, 2000). Such phenomenon, in which third-party animals gather information through the interception of signals, as been termed eavesdropping (McGregor, 1993).

Eavesdropping can be valuable to bystanders, since they can extract valuable information without the costs associated with engaging in direct interactions with other animals (Danchin et al., 2004). Thus, it is a cost-effective and simple way of learning about their social environment. This form of social learning has mainly been studied in mating and territorial contexts (Valone, 2007). For instance, when searching for a mate, females can either eavesdrop on male-male contests (Amy et al., 2008; Doutrelant & McGregor, 2000; Ophir & Galef, 2003; Otter et al., 1999) or male-female interactions (Alonzo, 2008; Dugatkin & Godin, 1992; Galef & White, 1998). Observing these can influence the females' mating decisions and also how they direct their extra-pair behaviour (Mennill, Ratcliffe, & Boag, 2002).

Males can also acquire information about their neighboring males through eavesdropping, mainly to determine their fighting abilities (Valone, 2007). This way, bystanders might decrease the risk of injury when engaging in such contests by adapting their behaviour towards their rivals (McGregor, 1993). In the Siamese fighting fish, males eavesdrop on aggressive interactions between other males. They differently responded to a territorial intrusion performed by the winner or loser of an observed interaction. Indeed, bystanders took longer to approach and display to seen winners than to seen losers (Oliveira et al., 1998). Green swordtails also modulate their behaviour towards winners and losers of observed interactions (Earley & Dugatkin, 2002). Bystanders were less likely to initiate a contest with seen winners and the probability of them winning these encounters was significantly decreased.

Male Great tits also eavesdrop on vocal contests between nearby rivals by decreasing the number of songs produced towards a perceived loser (Peake et al., 2001). In a similar study made in nightingales, bystanding males spent more time singing to

perceived winners of simulated interactions. They also used more songs and the latency to approach them decreased (Naguib et al., 1999).

The use of extracted information, through eavesdropping, has also been studied in male canaries (Amy & Leboucher, 2009), the rainbow trout (Johnsson & Åkerman, 1998), the golden hamster (Lai et al., 2014) and the *Astotilapia burtoni* (Grosenick et al., 2007). Particularly on this last case, bystanders were able to inference dominance hierarchies just by the observation of aggressive interactions between neighboring males.

Although animals can gather information through social learning, they also directly interact with their social environment (Dall et al., 2005). Thus, one might expect that animals can make decisions about social parameters by integrating their personal experiences with the eavesdropped information. For instance, experiencing a victory or a defeat in an aggressive event can influence an animal's posterior aggressive behaviour (Rutte et al., 2006). In many species, individuals that experience a victory have an increased probability of winning a second contest while experiencing a defeat increased the chance of losing a subsequent interaction (Rutte et al., 2006). These are called winner and loser effects and have been reported in several species (Khazraïe & Campan, 1999; Rui F Oliveira et al., 2011; Oyegbile & Marler, 2005; Whitehouse, 1997). Also, a study made with golden hamsters has demonstrated that such fighting experiences can impact on how animals use socially gathered information about their rivals (Lai et al., 2014). Male hamsters, with no previous fighting experience, spent more time near the winners of seen interactions. However, when males experienced a defeat before observing a contest between two demonstrators, they spent less time near the seen winners. Such a difference in response might suggest that previous fighting experiences can influence how animals use the information gathered through eavesdropping.

Here, we aimed to determine if zebrafish males eavesdrop on aggressive contests between neighboring males and, if so, whether experiencing a prior victory or defeat can influence how bystanders use the gathered information.

Zebrafish is a social species, that lives in mixed-sex shoals, exhibit territorial behaviour and can establish dominance hierarchies (Spence, 2007). Also, their aggressive behaviour has been well characterized (Oliveira et al., 2011).

Zebrafish is also a model organism, due to its high fecundity rate, small size, high genetic homology to humans and transparent embryos and larvae that facilitate the study of its development (Stewart et al., 2014). Therefore, by being highly studied in several areas of biology it has an extensive "genetic tool kit" available (Oliveira, 2013). Due these advantages, zebrafish is starting to be considered a good model in which to study social cognition.

A recent study has used zebrafish to measure a bystanders' attentiveness towards an aggressive interaction between two males (Abril de Abreu, *unpublished data*). In this work, attentiveness was measured using the time bystanders' spent near the demonstrators' aquarium when aggressive interactions took place, as well as their directionality in the tank. It was shown that bystanders spent more time near the demonstrators' aquarium, also spending more time facing them. These same results were not obtained when the social stimulus is present but no interaction occurs.

As so, we hypothesized that bystanders adjust their behaviour towards two rivals, after observing an aggressive interaction, with a clear outcome between them. We also investigated if rival assessment could be affected by a previous social experience, in this case, being a loser or a winner on a prior agonistic interaction.

METHODS

In this experiment, we wanted to determine the existence of eavesdropping in zebrafish, in the assessment of other males fighting abilities, after the observation of a territorial dispute. As so, bystanders were allowed to observe an interaction between two demonstrator fish, therefore observing its outcome. Afterwards, the bystanders' swimming behaviour was tracked. Also, the bystanders' dominance status was manipulated, prior to observing these aggressive encounters.

Animal Housing

We used zebrafish males, from the AB strain, reared in laboratory facilities. They were kept in 50 x 25 x 30 cm tanks, in shoals of 30 individuals, and their environment was enriched with small rocks, artificial plants and also females of several ages (5 per tank). These animals were kept in a 12:12 L/D cycle (from 07:30 AM to 07:30 PM), with a room and water temperature of 25^{o} C . They were fed in the morning with crushed TetraMin tropical fish food flakes and with live food (*Artemia salina*) twice in the afternoon. The 37,5 L aquaria were filled with aged tap water and one third of it was changed every eight days.

Experimental Set up

The main experimental setup was composed by four demonstrator tanks (30 x 15 cm) placed in front of four bystander arenas (13 x 13 cm), both with a water height of 9 cm (**Figure 2.1**). The demonstrator tanks were divided in two by a transparent partition and then divided again by an opaque removable partition. These were attached to strings to decrease experimenter direct manipulation. The bystanders were placed in the bystanders' arenas while the demonstrator fish were placed in the demonstrator tanks, on the nearest half to the bystanders' arenas. The other half was empty, increasing the distance between the fish and experimenter.

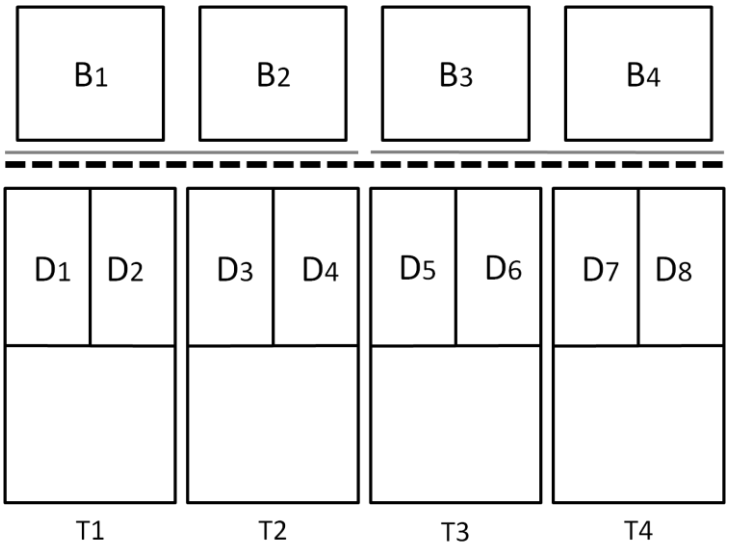

Figure 2.1. Main Experimental Set up. B1 to B4 corresponds to the bystander arenas. D1 to D8 corresponds to the demonstrator fish, placed in the demonstrators' tanks (T1 to T4). Grey lines represent removable opaque partitions while the dotted lines represents the one-way mirror.

Between the bystanders' arenas and demonstrator tanks, a one-way mirror (30 x 18 cm). was placed as well as removable opaque partitions. The one-way mirror allowed the bystanders to observe the demonstrators while preventing them from visually contact with the bystanders. Also, the removable opaque partitions allowed us to block the bystanders' visual access to the demonstrators when needed. The set up was illuminated by a 60 cm long LED light, placed on top of the tanks, and all water was constantly oxygenated (excluding the time when bystanders were being tested).

The main experimental set up was assembled on top of a LED infra red table (62x62 cm) and a metallic structure (75 x 29 x 42,5 cm), holding four top-view cameras, was placed above it in order to record the bystanders behaviour during the experiment. A second camera (front-camera) placed in front of the demonstrators tanks was used to record demonstrators' interactions and post-interaction periods (*see Experimental Protocol*). During the experiment, the existence of black curtains minimized the contact between the fish and the experimenter.

Bystanders were submitted to a territorial dispute before being placed in the experimental setup described above. This occurred in 15 x 15 cm arenas, with a water height of 9 cm, divided in half by opaque removable partitions attached to strings. These prevented visual and physical contact between the pair allowing, though, chemical contact. Also, there was no contact between fish of different pairs. These arenas were placed inside a bigger tank (49,5 x 25 cm), also with a water height of 9 cm, containing a mixed-sex shoal of 30 individuals (**Figure 2.2**).

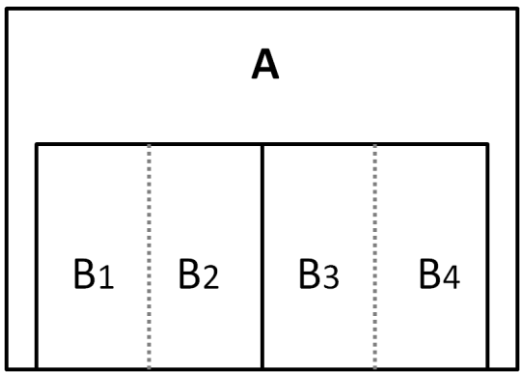

Figure 2.2. Manipulation of dominance arenas. Two arenas were placed within a larger tank comprising a shoal of zebrafish males (A). Future bystanders (B1 to B4) interacted in these arenas so that their dominance status could be determined.

The presence of conspecifics decreased stress related behaviours that the future bystanders experienced when they were placed into these arenas. The floor of the bigger tank was covered by grit, and water was constantly oxygenated. A front camera recorded the manipulation of dominance status disputes.

Experimental Protocol

The protocol lasted three days:

Day 1 - Four fish were taken from the general stock. Each of them was placed in one half of the manipulation of dominance arenas (**Figure 2.2**), and then left to habituate for 24 hours. The fish within the same pair were not familiar to one another, since they came from different shoals.

Day 2 - On the following day, the opaque partitions were removed. The fish were left to interact, while being recorded by the front-camera, until they reached resolution, that is, until a clear dominance between them was established. This was easily identifiable through observation, since winners aggressively chase, bite and strike losers, while these flee and adopt a submissive posture (Oliveira, 2011). After this, the fish were separated once more and the recorded interaction was analyzed to determine the position of the winner and loser at the time of separation. The winners became the dominant bystanders while the losers were referred to as subordinate bystanders.

Dominant and subordinate animals were placed in the corresponding bystander arenas of the main experimental set up. The order in which dominant and subordinate bystanders were placed was randomized. Two bystanders were subjected to the control treatment while the other two were submitted to the experimental treatment. As so, in each session, four different conditions were applied: Experimental Dominant (ED); Experimental Subordinate (ES); Control Dominant (CD) and Control Subordinate (CS). Although the bystanders' position was randomized, because of the long protocol and the possibility of simultaneously record the behaviour of two subjects, with the top-cameras, bystanders were tested in pairs. That is, the same treatment was applied for two adjacent bystanders. For instance, B1 with B2 and B3 with B4 (**Figure 2.1**)

(**Table2.1**). Also, six fish were placed in demonstrator tanks. They were matched for body length within the pair (assessed visually). All the fish were fed with *Artemia salina* after being placed in the main set up. The bystanders were left to habituate overnight to the demonstrators.

Table 2.1. Randomization of the four groups (CD, CS, ED and ES) throughout the bystander arenas (B1 to B4) and all the experimental sessions. CD - Control Dominant group; CS - Control Subordinate group; ED - Experimental Dominant group; ES -Experimental Subordinate group.

SESSION	B1	B2	B3	B4
1	CD	CS	ED	ES
2	ES	ED	CD	CS
3	ED	ES	CS	CD
4	CS	CD	ES	ED
...	...	...	...	...

Day 3 - The eavesdropping experiment started with the recording of a 30 minutes behavioural baseline (**BL Timepoint**, **Figure 2.3**). Because we wanted to determine if bystanders were capable of gathering and use eavesdropped information, the fish subjected to the experimental treatment were allowed to observe a fight between the demonstrators, lasting 30 minutes (**IO Timepoint, Figure 2.3**). The control bystanders were not allowed to observe these fights and, therefore, the opaque partitions were placed between them and the demonstrators' tanks during this period of time (**IN Timepoint**, **Figure 2.3**). Both the control and experimental bystanders' behaviour was recorded using top-view cameras, while the demonstrator fish interacted**.** The interactions were recorded with a second synchronized front camera. After the separation of the demonstrators and the removal of the opaque partitions previously placed in front of the control bystanders, the subjects' behaviour was recorded for another 30 minutes (**AI Timepoint, Figure 2.3**). At the same time, the demonstrators post-fight period was also recorded.

Since the control bystanders did not observe the interactions between demonstrator fish, a blackout period for experimental bystanders was required so all bystanders (experimental or control) visually contacted with the demonstrators for the same amount of time. Therefore, opaque partitions were placed, for 30 minutes, between the

experimental bystanders and the demonstrators before the interactions took place (**BO Timepoint, Figure 2.3**). Control bystanders did not experience this blackout period, therefore being able to observe the demonstrators for the corresponding period of time. After this, the opaque partitions were removed and, once more, bystanders' behaviour was recorded for an additional 30 minutes (**BI Timepoint, Figure 2.3**). This second sampling period was required to determine if the blackout period we subjected the experimental bystanders to, affected their subsequent behaviour.

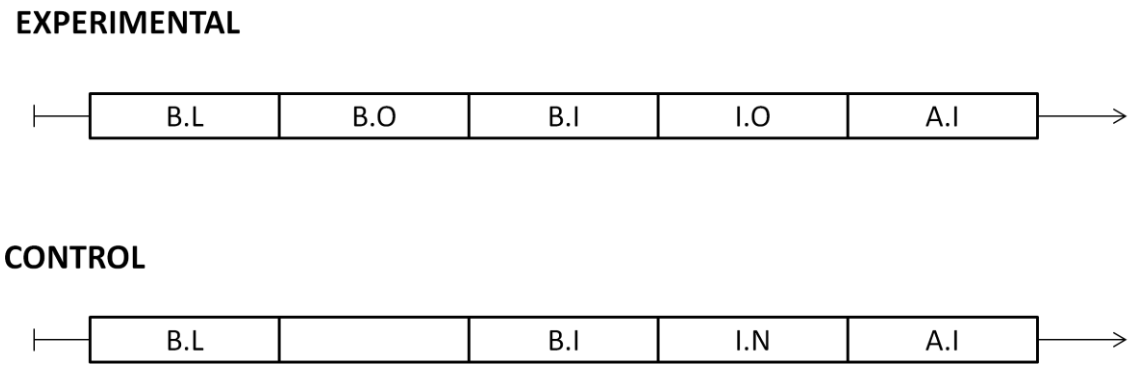

Figure 2.3. Experimental and Control treatments represented in a timeline. Each rectangle corresponds to a 30 minute period of time. BL - Baseline; BO - Blackout; BI - Before Interaction; AI - After Interaction; IO - Interaction observed; IN - Interaction not observed.

These experiments took place between December 5th, 2013 and April 2nd, 2014. The social eavesdropping experiment took place from 9 am to 2:30 pm, while the manipulation of dominance disputes occurred between 3 pm and 5 pm. We used a total of 72 males, ranging in age from nine to twelve months old, as bystanders (n=19 for the ED group; n= 18 for the ES group; n=18 for the CD group and n=17 for the CS group). 30 males with their ages ranging from eight to eleven months old were used as demonstrators. Bystanders were only used once but demonstrators were reused several times.

Video Recording

The bystanders' videos were recorded using top-view mini CCTV 300B cameras, with 420 TVL, while the manipulation of dominance disputes and demonstrators' interactions and post-interactions periods were recorded using a SONY Handycam DCR - SR58E camera. Both of these videos had a frame rate of 25 frames per second.

Video and Behavioural Analysis

The post-interaction videos of the demonstrators (i.e. AI timepoint), were used to measure the fish. For each demonstrator, five frames were isolated and fish were measured using IMAGE J 1.46r. Winners and losers had a standard length of 2.52 ± 0.04 cm and 2.48± 0.04 cm, respectively.

The bystanders' movement within their tanks, from a top view perspective, were tracked using a custom-made software (Python™). Firstly, the arena luminosity was increased to diminish shadows that could disrupt the tracking of the bystander's movements. Afterwards, an area inside the arena was defined and the fish position within its limits was registered frame by frame. The definition of this area considered the distortion produced by water depth and comprised the bottom of the arena (13 x 13 cm) as well as the demonstrators' observation side in which the contrast between the illuminated background and the fish was high (**Figure 2.4**). The black outer sides were excluded from the tracking analysis due to low contrast.

For each frame, the coordinates in pixels of the head, centroid and tail were extracted into data files, enabling the determination of the fish position within the defined area. In order to detect possible tracking errors, the total time spent at each position was plotted in 2D heatmaps and spatial histograms.

The generated data files were imported to MATLAB (MathWorks®) and behavioural variables were determined using a custom-made script. For each bystander, in all timepoints, we extracted the total distance(m) travelled by the fish and its average speed (m/s) in all its arena. The total distance and average speed were used to measure the level of activity of each bystander, in each timepoint.

To determine if bystanders altered their swimming behaviour towards the demonstrators of a seen (experimental) or unseen (control) interaction, for the AI timepoint, a region of interest (R.O.I) was defined. This region corresponded to 25% of the tank in the area closest to the demonstrators tank (13 cm width and 3.25 cm length). Within this region we defined two sides, by dividing it in half (**Figure 2.4**). Therefore, each side corresponded to the closest area near the winner or the loser of the previously seen (experimental) or unseen (control) interaction, within the bystander tank. Thus, each area corresponded to 12,5% of the bystander arena.

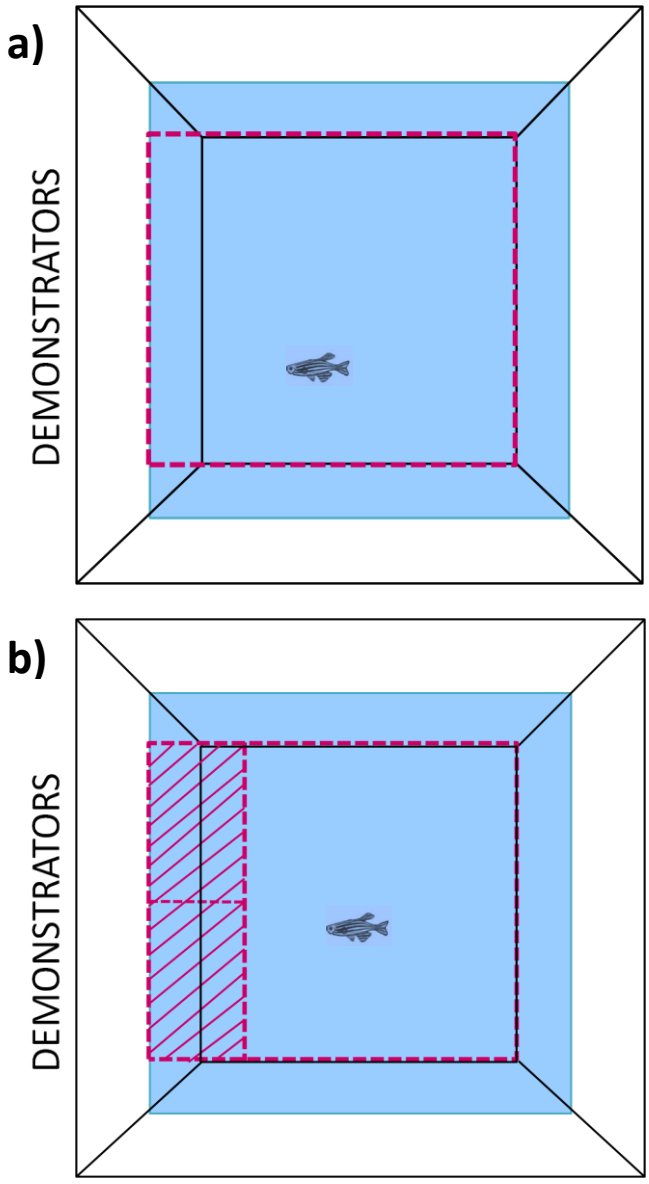

Figure 2.4. Areas within the bystanders' arenas defined to extract behavioural measures.

a) This area comprised almost all the bystander arena. Within it, the fish was tracked for all the recorded timepoints.

b) The filled region within the previously defined area corresponds to the region of interest (R.O.I). It is the closest region to the demonstrators, comprising 25% of the bystander arena. This region was divided in half - the winner's side and the loser's side.

Considering this, for the winner and loser side within R.O.I, we extracted the following variables (AI timepoint): (1) Total time (s); (2) Total Distance (m); (3) Average Speed (m/s).

Measuring the time each bystander spent in R.O.I as been shown to be a good measure of attentiveness in zebrafish when demonstrators interact (Abril de Abreu, *unpublished data*). Indeed, zebrafish males spent more time in the region closest to the demonstrators when an interaction is occurring. In this study we were measuring attentiveness towards each demonstrator, the winner and loser of a previous interaction. We know that zebrafish males demonstrate territorial behaviour (Pérez-Escudero, Vicente-Page, Hinz, Arganda, & de Polavieja, 2014) mainly by chasing away intruders (Spence & Smith, 2005). Since the bystander tank can be perceived by the subject as its defensible territory, one might expect that bystanders will be more attentive towards potential intruders, therefore spending more time in the limits of its own territory.

We calculated a preference score for each bystander as the total time spent in the winner's side of R.O.I (s) over the total time spent in both sides of R.O.I., ranging between 0 and 1. Values below 0.5 indicate that the bystander spent more time in the loser side of R.O.I while values above it indicate that fish spent more time in the winner side.

Statistical Analysis

Of the 72 pairs tested, 4 were removed due to being considered as extremes (detected using an interval of ±3 standard deviations) in the analysis (1 for each group).

To determine the effect of the blackout, we conducted a repeated measures ANOVA on the total distance (m) and average speed (m/s), between the BL and BI timepoints. Treatment (Experimental or Control) and status (Dominant or Subordinate) were used as independent variables.

To detect the use of eavesdropped information by the observers, for the AI timepoint, we conducted a factorial ANOVA on the calculated preference score, total distance (m) and Average Speed (m/s) for all arena. We also performed a repeated measures ANOVA for the total distance (m) and average speed (m/s) in the winner and loser sides of R.O.I. Once more, we used treatment and status as independent factors.

Data normality was tested using the Shapiro-Wilkes test. For data violating the normality assumption, we used either a square-root or a logarithmic transformation. In this case, for the AI timepoint, the total distance(m) and average speed (m/s) in all the arena, as well as total Distance (m) and average speed (m/s) in winner and loser's side of R.O.I.

We used the STATISTICA (version 12), Statsoft Inc., software to analyze the data.

Ethical Note

No fish was injured as a result of the expression of aggressive behaviour. Used animals returned to the stock and were re used in other pilot studies. This experiment was conducted under the permit for animal experimentation #008955 issued by Direção Geral de Alimentação e Veterinária.

RESULTS

No influence of the blackout period in the total distance (For BL vs. BI timepoints, ED: 60.36 ± 4.85 m vs. 5498 ± 3.02 m, N=18; ES: 73.45 ± 5.95 m vs. 60.63 ± 6.59 m, N=17; CD: 59.72 ± 4.65 m vs. 50.77 ± 3.23 m, N=17; CS: 63.01 m ± 7.48 vs. 51.46 m ± 4.85, N=16) or average speed was found. (BL vs. BI: ED: 0.032 ± 0.002 m/s vs. 0.030 ± 0.002 m/s; ES: 0.039 ± 0.003 m/s vs. 0.032 ± 0.003 m/s; CD: 0.032 ± 0.002 m/s vs. 0.027 ± 0.002 m/s; CS: 0.033 ± 0.003 m/s vs. 0.028 ± 0.002 m/s).

However, an overall significant decrease in these variables was detected (Total Distance: ANOVA, $F_{1,64}$: 25.711, P=0.0000; Average Speed: ANOVA, $F_{1,64}$: 23.147, P=0.00001)(**Figure 2.5**). Indeed**,** the total travelled distance by the subjects of all groups

(N=68) decreased from 65.00 ± 2.90 meters, in the BL timepoint, to 54.51±2.30 meters in the BI timepoint. The average speed was also higher in BL (0.034 ± 0.001 m/s) rather than BI timepoint (0.029 ± 0.001 m/s).

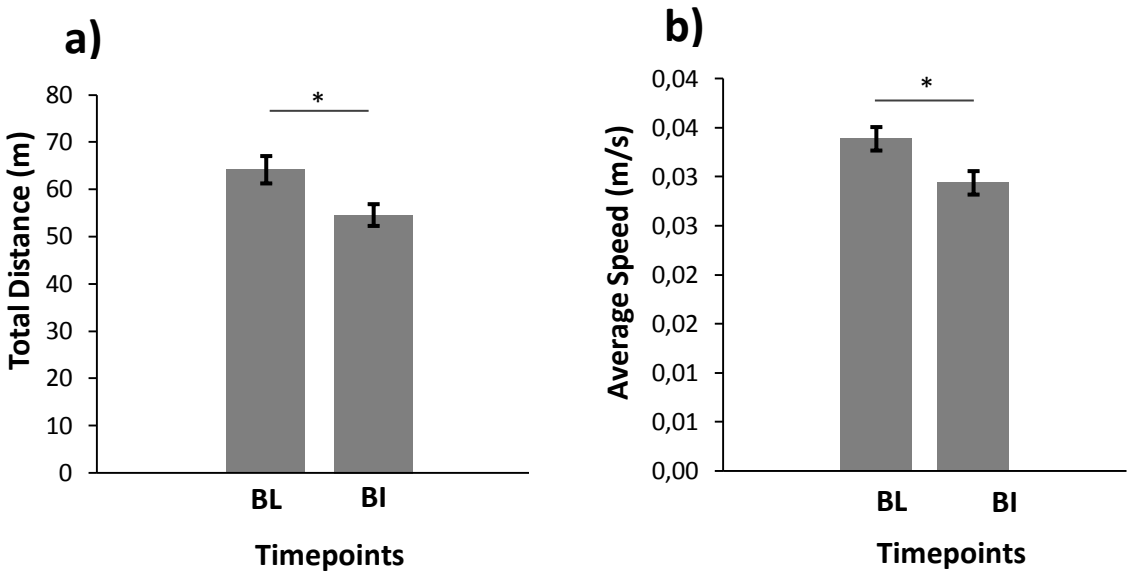

Figure 2.5. Comparison between the total distance (a) and average speed (b) of the bystanders, between the BL and BI timepoints.

For the AI timepoint, no differences in the total distance (ED: 46.84 ± 3.73 m; ES: 56.46 ± 4.23 m; CD:53.70 ± 2.35 m; CS: 53.38 ± 3.63 m) and average speed (ED 0.025 ±0.002 m/s; ES: 0.030 ± 0.002 m/s; CD: 0.029 ± 0.001 m/s; CS: 0.029 ±0.002 m/s), for all the arena, were found between the four groups (**Figure 2.6**).

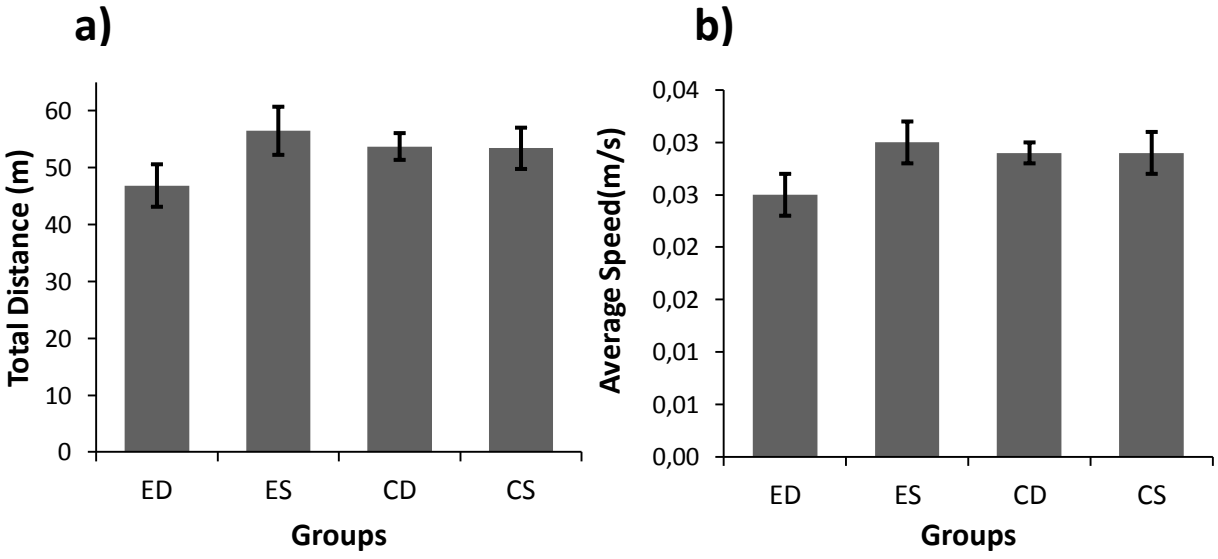

Figure 2.6. Bystanders' total travelled distance (a) and average speed (b), for the AI timepoint, across all the four groups. No significant differences between groups were found for these variables.

When comparing the winner and loser sides of R.O.I, we also could not found an influence either treatment or dominance status on the preference score values, total distances travelled by the fish or average speeds (**Figure 2.7**).

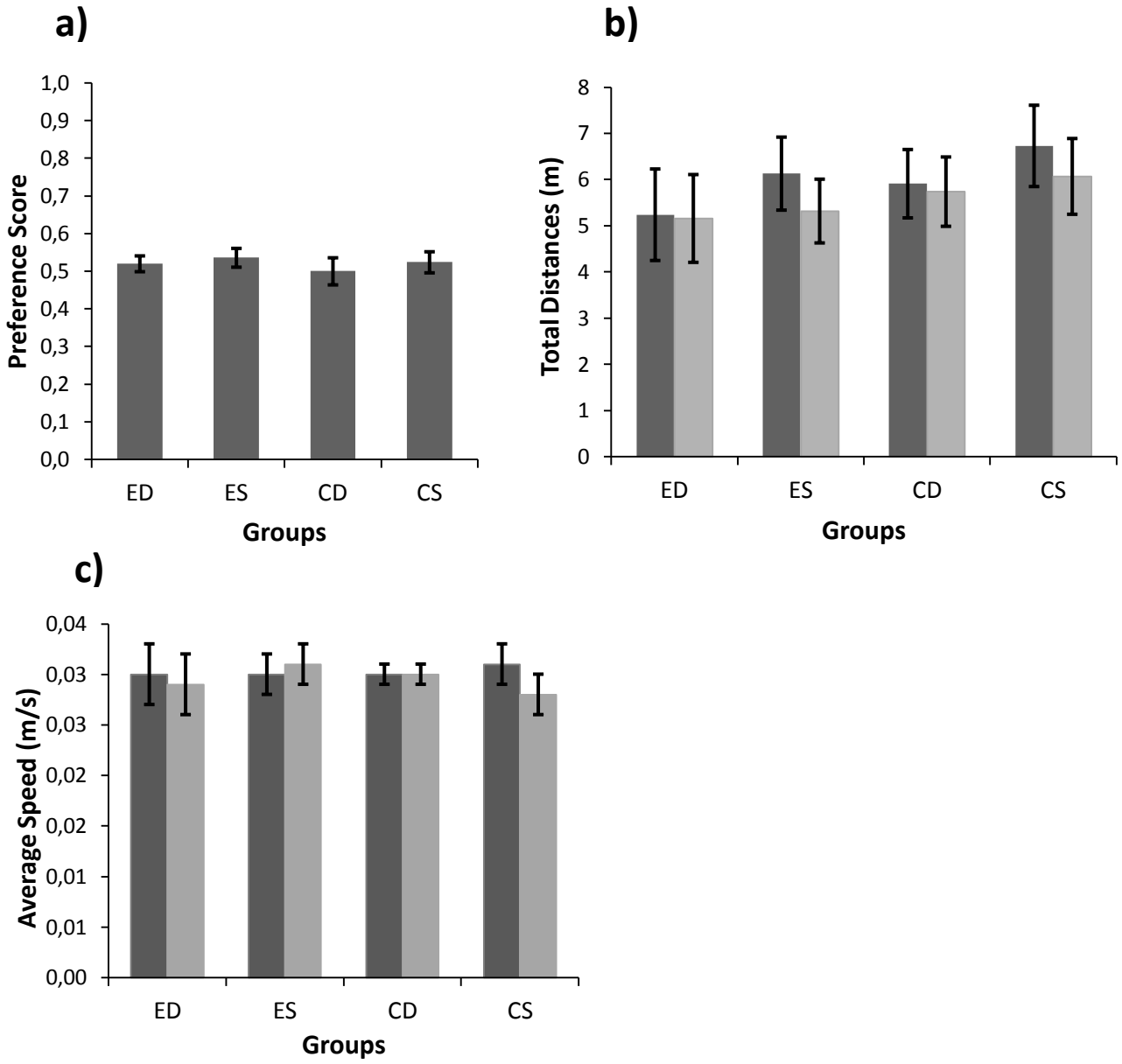

Figure 2.7. Comparison between winner and loser sides of R.O.I., for the AI timepoint, across all groups. (a) Preference score, ranging between 0 and 1 (ED: 0.52 ± 0.02; ES: 0.54 ± 0.03; CD: 0.50 ± 0.04; CS:0.52 ± 0.03). **(b) Total distance (m)** travelled by the fish, in the winner (Dark grey) (ED: 5.24 ± 0.99 m; ES: 6.13 ± 0.79 m; CD: 5.912 ± 0.74 m; CS: 6.73 ± 0.88) and loser side (Light grey) of R.O.I.(ED: 5.16 ± 0.95 m; ES: 5.32 ± 0.69 m; CD: 5.74 ± 0.75 m; CS: 6.07 ± 0.82 m).**(c) Average Speed (m/s)** in the winner (ED: 0.030 ± 0.003 m/s; ES: 0.030 ± 0.002 m/s; CD:0.030 ± 0.001 m/s; CS: 0.031 ± 0.002 m/s) and loser side of R.O.I. (ED: 0.029 ± 0.003 m/s; ES: 0.031 ± 0.002 m/s; CD: 0.030 ± 0.001m/s; CS: 0.028 ± 0.002 m/s).

DISCUSSION

In this study, we aimed to understand if zebrafish males that had observed an agonistic interaction between two potential rivals could gather and use information about their fighting abilities and if dominance status influenced the use of the gathered information. To do so, we measured the subjects' preference towards seen or unseen winners and losers, by calculating a preference score within R.O.I, as well as the total distances and average speeds, both in all the arena and in each side of R.O.I.

Here, we found that bystanders did not demonstrate any preference towards either winners or losers (seen or unseen). Due to this, we also failed to determine if prior experience (i.e. a prior defeat or victory in a contest) influenced the use of the socially acquired information.

These results can lead to two possible explanatory hypotheses. The first one is that this form of social learning is absent in the zebrafish. This seems very unlikely given the presence of eavesdropping in several species (Canaries: Amy & Leboucher, 2009; Golden Hamsters: Lai et al., 2014; Nightingtales: Naguib et al., 1999; Great Tits: Peake et al., 2001), including other fish species (Green swordtails: Earley & Dugatkin, 2002; Tilapia: Grosenick et al., 2007; Rainbow trout: Johnsson & Åkerman, 1998; Siamese fighting fish: Oliveira et al., 1998). The fact that eavesdropping has been detected in so many species and in different contexts might be an indication that this form of social learning is a widespread phenomenon. Indeed, eavesdropping on others' signaling interactions can bring several advantages to both males and females. On one hand, males can gather information about other males without the risk of injury associated with a direct contest (McGregor, 1993) . On the other hand, females can evaluate potential mates either by eavesdropping on male-female or male-male interactions, without the energy investment associated with trial-and-error tactics (Danchin et al., 2004). Therefore, using eavesdropping can be a simpler and effective way of navigating in the social environment (Valone, 2007).

Another reason why this first hypothesis seems unlikely is that an audience effect has been detected in this species (*see paper B*), in an aggressive context. Indeed, zebrafish males adjusted their signaling behaviour when conspecifics were present. The fact that the presence of an audience can have an impact on agonistic behaviour in males, is an indication that nearby conspecifics might eavesdrop on others' signals. It is therefore expected that zebrafish can use eavesdropping as a mean of gathering information.

Therefore, it is more likely that we were not able to detect the occurrence of this phenomenon. Our experimental set up might not have been the best one to detect the use of gathered information by the observers. Indeed, we only used attentiveness towards winners and losers, expressed by time spent in each side of R.O.I, which was used to calculate the preference score, as well as total distances and average speeds, to measure the use of gathered information. However, no direct contact was promoted between bystanders and demonstrators. Thus, one might speculate that observers could gather information about their rivals and only use it when a direct interaction occurs, by altering their aggressive behaviour towards the eavesdropped competitor.

Another possible reason for why our experimental set up might not have been the best to demonstrate the occurrence of eavesdropping is that, subjects could observed both demonstrators from all the points of the bystander arenas. That is, for each position within their arena, males could visually contact with either the winner or loser and, therefore, be attentive to both.

In this experiment, we also detected high levels of stress in our subjects, as indicated by the expression of erratic movement. Indeed, when comparing BL and BI timepoints, our results demonstrated that, for all groups, the total distance travelled by the bystanders as well as their average speed decreased. The fact that we had to manipulate cameras and cables near the experimental set up, at the beginning of each session might have increased the fish stress levels, therefore promoting an overall increase of total distance an average speed in the first timepoint (BL).

Considering this, we emphasize that future work should account for these all the problems discussed above. For instance, a forced choice apparatus with a greater distance between demonstrators could be a good option to re-test the occurrence of eavesdropping in zebrafish, using the same variables used here. Another possible approach would be to promote territorial intrusions from each demonstrator in order to evoke a direct behavioural response from the bystander towards winners and losers. In this case, the bystanders' agonistic behaviour could be measured to determine if the response towards a seen winner differs from the response towards a seen loser. Finally, a new approach would need to consider the decrease of stress levels on the bystanders, for instance, by decreasing even more its exposure to the experimenter or by using a less stressed zebrafish strain such as the Tubingen.

3. Paper B

The effect of an audience in zebrafish (*D.rerio*) agonistic interactions

Animals communicate through the interchange of signals, therefore acting as reciprocal signalers and receivers(McGregor & Peake, 2000). However, communication is not only restricted to dyadic interactions, since signals exchanged within the dyad are also available to nearby individuals, within the range of signal transmission. Therefore, communication occurs in the form of a network comprising signalers, receivers and bystanders (McGregor & Peake, 2000). These third-party elements can detect and intercept these conspicuous signals, which can be potentially relevant to them, in a phenomenon known as Eavesdropping (McGregor, 1993). As so, animals can extract and use this information to their benefit, increasing their chances of survival and reproductive success.

Several studies have reported the existence of this phenomenon, both in the mating (Amy et al., 2008; Doutrelant & McGregor, 2000; Dugatkin & Godin, 1992; Ophir & Galef, 2003; Otter et al., 1999) and territorial context (Amy & Leboucher, 2009; Earley & Dugatkin, 2002; Naguib et al., 1999; Peake et al., 2001). For instance, in the Siamese fighting fish, males differently respond to a territorial intrusion performed by a winner or a loser of a previously observed interaction (Oliveira et al., 1998). Indeed, their latency to approach and display increased towards seen winners.

The presence of third-party elements, capable of detecting and intercepting signals not directly sent to them, can lead to a situation in which the presence of such individuals could potentially influence the signaling behaviours of others. Thus, animals might alter their signaling behaviour in the presence of potential eavesdroppers, either by manipulating the conspicuousness or intensity of their signals, a phenomenon named audience effect (Marler et al., 1986). Therefore, bystanders within communication networks are simultaneously influencing the interactions of others as well as being influenced by the signals they eavesdrop on (Earley & Dugatkin, 2002).

Indeed, several studies have reported the existence of such effects. For example, the presence of bystanders can influence the mating decisions of observed animals. In Atlantic mollies, males prefer larger over smaller females. However, when a by standing competitor is nearby, males spent an equal amount of time with both females, thus

losing their innate preference (Plath & Schlupp, 2008) . Similar results were also found in cave mollies (Blum et al., 2008).

The sex composition of an audience can be an important factor that influences the modulation of signalers' behaviour. This is the case of the Siamese fighting fish, in which males decrease their aggressive signaling towards a competitor, when a female, rather than a male audience, is present. They increase the number of behaviours both used in aggression and courtship (Doutrelant et al., 2001). Also, in male field crickets, aggressiveness towards competitors increases when an audience, either composed by a male or a female, is present. Yet, the number of victory displays performed after winning a contest only increases in the presence of a male audience (Fitzsimmons & Bertram, 2013).

Prior social exposure to males in an audience can also affect aggressive signaling in male-male contests (Dzieweczynski & Perazio, 2012). In fact, in the presence of an unfamiliar male audience, Siamese fighting fish males increase the time spent displaying towards an opponent and the bystander male, also spending more time near the audience tank. In this species, the time of presentation of an audience can also influence the male-male interactions. When males were pre-exposed to a bystander male (i.e before an aggressive encounter), the latency for the first bite decreased (Matos et al., 2003). This effect is called aggressive priming; although the bystander was not present at the time of the interaction, male still adjusted their signaling behaviour, by increasing their aggressiveness towards its rival (Matos et al., 2003).

Here we were interested in determining the existence of audience effects in the zebrafish. They are highly social animals, that live in mixed-sex groups (Spence et al., 2008) and can establish dominance hierarchies through the expression of territorial behaviour (Spence et al., 2008). Their aggressive behaviour in contests has also been described in detail (Oliveira et al., 2011). Also, zebrafish is an organism used in several fields of biology such as development and neuroscience (Oliveira, 2013), due to its small size, high reproductive rates (Spence et al., 2008) which allows an easy accommodation. Their embryos and larvae are transparent enabling a detailed study of their development. Their genetic homology to humans is also high (Stewart et al., 2014).

Thus, by being a model organism, zebrafish can be used to understand the cognitive processes underlying social behaviour.

Therefore, in this study, we aim to determine if the visual presence of an audience during a contest between males promoted a change on their signaling behaviour. We were also interested in determining if pre-exposure to an audience before a contest influences signaling behaviour during the interaction.

METHODS

Animal Housing

We used zebrafish males from the AB strain, reared in laboratory facilities, with ages ranging between 7 to 9 months old. They were kept in 50 x 25 x 30 cm tanks, in groups of 30 males, and their environment was enriched with small rocks, artificial plants and also females of several ages (five per tank). These animals were kept in a 12:12 L/D cycle (from 07:30 AM to 07:30 PM), with a room and water temperature of 25^{o}C . They were fed in the morning with crushed TetraMin tropical fish food flakes and with live food (*Artemia salina*) twice in the afternoon. The 37,5 L aquaria were filled with aged tap water and one third of it was changed every eight days. This experiment occurred between June and July 2014.

Treatments

Half of the sample was exposed to a conspecific audience during the aggressive signaling event while the other half was not. We also tested for agressive priming in zebrafish by pre-exposing half of our sample to an audience while the other half was not pre-exposed. Therefore, there were four treatments, with two independent factors (exposure to audience and pre-exposure to audience) (**Table 3.1**): (AA) Audience present both before (pre-exposure) and during (exposure) the interaction; (AN) Audience before (pre-exposure) but not during (no exposure) the interaction; (NA) Audience not present

before (no pre-exposure) but present during (exposure) the interaction; (NN) Audience absent both before (no pre-exposure) and during (no exposure) the interaction.

Factors		Exposure during interaction	
		Yes	No
Pre-exposure	Yes	AA	AN
	No	NA	NN

Table 3.1. Presence of an audience of conspecifics before or during an agressive event according to treatment. A - Audience Present; N - No audience

We used a total of 96 pairs of males, (24 per treatment). To avoid any potential effect of past experience, each pair was only used once and within each pair, males were unfamiliar to each other (i.e. they came from different home tanks).

Audience Composition

In terms of the audiences' sex composition, we used a mixed-sex audience since zebrafish is a highly social species in which shoals include both males and females (Spence *et al*., 2008) . We decided to use shoals of four individuals (2 males and 2 females) as an audience for two reasons: (1) Zebrafish males did not have a preference for a specific shoal size (Ruhl & McRobert, 2005); (2) Our audience tanks (**Figure 3.1**) had a 15 x 15x 9 cm water volume, therefore ideally accommodating no more than four fish.

Audiences were left to habituate to the experimental set up for three weeks prior to the experiment. We have done this so that dominance relations between individuals would stabilize before the experiment started, therefore diminishing the probability of occurrence of aggressive contest within the audience at the time of testing. The same audiences were used throughout the experiment.

Experimental Set up

The experimental set up was composed by four same size (15 x 15 cm) arenas and two 30 x 15 cm arenas (**Figure 3.1**). The longer ones were the audience tanks (so called **A** and **B**) in which the audience elements were placed. They remained in only half (15 x 15 cm) of these 30 x 15 cm arenas since on the other half, water was oxygenated and filtered. A light bulb was placed on top of the audience tanks.

On the side of each, we had two arenas (**1**, **2**, **3** and **4**), in which we placed our subjects, physically (but not chemically) separated by opaque partitions. Between the arenas and the audience tank, one-way mirrors were placed (**Figure 3.1**, **blue lines**), in such a way that subjects could visually contact with the audience but not the other way around. One removable opaque partition was placed between each arena and the audience tank, so that we could manipulate the subjects' visual access to the audience (**Figure 3.1, red dotted lines**).

To avoid any experimenter effects, we had a 30 cm high white protective barriers around the fighting arenas, with a 30 cm height (**Figure 3.1, green lines**), with an opening which allowed us to record the subjects' behaviours.

Experimental Protocol

The protocol was divided in two distinct periods, for all treatments: (1) the Habituation period, which lasted 24 hours; (2) and the Fighting period, with a 30 minutes duration.

In the AA and AN treatments, subjects habituated to the experimental set up while being pre-exposed to the audience, that is, no opaque partitions were placed between them and the audience tank, during this period (**Figure 3.1**). The NA and NN pairs habituated without visual contact with the audience.

After this period, the aggressive interactions between subjects took place. In all treatments, the opaque partitions between subjects within the same arena were lifted.

In the AA or NA treatments, the visual contact with the audience was allowed - no opaque partition between the subjects and the audience tanks.

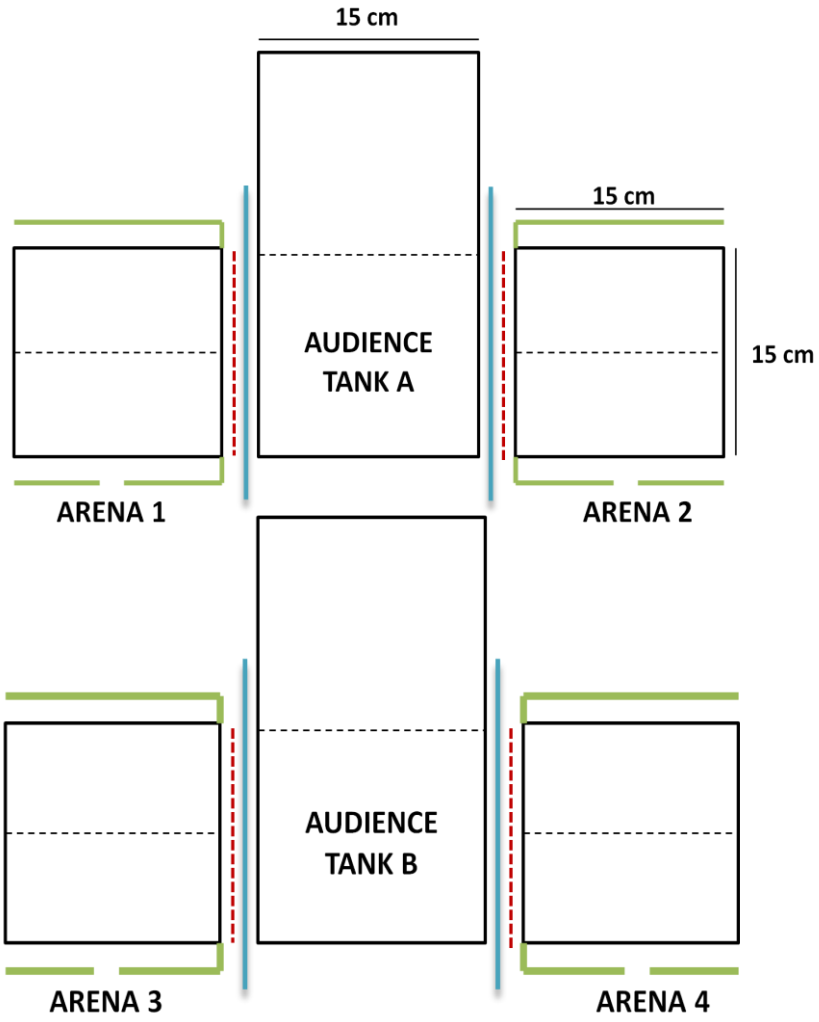

Figure 3.1. **Experimental Set up** – The blue lines represent one-way mirrors, the red dotted lines represent removable opaque partitions and the green lines stand for the barriers that protected the fish from the experimenter's presence.

Otherwise, subjects would interact without the presence of an audience, that is, with the opaque partitions between them and the audience tanks. The placing or removal of these partitions, right before the contest started, was also mimicked for treatments in which this did not occur. This was the case of the AA and NN treatments.

In each session, we applied the four different treatments (one for each fighting arena), in a balanced order, such that all treatments were applied in all fighting arenas, throughout the 24 sessions of this experiment.

At the end of each session, all subjects were anesthetized with a Tricane (MS222) diluted solution (1:25 ml), measured (cm) and weighted (g). The difference in standard length and total body weight within each pair was then calculated.

Video Recording and Behavioural analysis

In order to measure agonistic behaviours between zebrafish males a side view of the agressive interactions between subjects were recorded using a video camera (SONY Handycam DCR - SR58E camera, 25 frames / second).

For each pair of males the following variables were measured: (1) Latency to Approach; (2) Number of bites; (3) Latency until first bite; (4) Time in display; (5) Latency until first display event; (6) Time in chasing; (7) Latency until first chasing event. These behaviours were measured using an event recorder software - OBSERVER XT ^{TM} version 7, Noldus Inc., Netherlands.

The occurrence of displays is characteristic of an assessment phase of the interaction, that is, when no dominance relationship between individuals has been established yet (Oliveira et al., 2011). Chasing behaviour is typical of a post-resolution stage of the interaction, when a dominant individual actively chases a subordinate one (Oliveira et al., 2011). Biting can occur throughout the agonistic interaction, although being more prominent in the post-resolution phase (Oliveira et al., 2011). Dominance can also be established without the occurrence of the assessment phase (*see Results*).

We scored how many interactions included assessment (detectable through the occurrence of displays) and how many had a clear resolution point (i.e. with clear dominance established between males), also considering how many of these resolved after the assessment stage took place.

We calculated an escalation of aggression index for each pair as the time spent in Chasing over the time in display plus the time in chasing. This escalation index gives the proportion between the pre- and post-resolution phases within each interaction, and ranges between 0 and 1.

Statistical Analysis

Of the 96 pairs tested, 7 were excluded from the analysis since males did not interact (2 from the AA group; 4 from the AN group and 1 from the NN group). We conducted a two-tailed factorial ANOVA on the measured behaviours and Escalation Index of resolved interactions. Pre-exposure to an audience and the presence of an audience during the interaction were used as independent factors.

We also analyzed if differences in the variation of Body weight and standard length between pairs were found between groups, using a two-tailed factorial ANOVA with the same factors above as independent variables. It is important to state that the variation in body weight had two values classified as high extremes within the same group treatment (AA). However, the analyses including and excluding these values lead to the same conclusions (*see Results*). In the analysis above, data normality was tested using the Shapiro-Wilkes test and a square-root transformation was performed for data violating the normality assumption, in this case, the time spent in chasing.

The percentage of interactions with the assessment phase, as well as the number of resolved interactions were analyzed using Pearson's chi-square tests, in order to determine the influence of pre-exposure to an audience and exposure to it during the contest on these dependent variables. When necessary, multiple comparisons between groups were also performed with Bonferroni corrections ($\alpha = 0.05$). For the percentage of resolved interactions after assessment in relation to the total number of resolutions, we used a two-tailed Fisher's Exact Test. Also in this case, multiple comparisons followed by Bonferroni corrections ($\alpha = 0.05$) were made.

We used the STATISTICA (version 12), Statsoft Inc, software to analyze our data.

Ethical Note

No fish was injured as a result of the expression of aggressive behaviour. Weight and length measurments occured as breifly as possible and audience tanks (A and B) amonia and nitrates concentration was monitorized throught the experiment. Animals returned to the stock and were re used in other pilot studies. This experiment was conducted under the permit for animal experimentation #008955 issued by Direção Geral de Alimentação e Veterinária.

RESULTS

The variation of body weight between opponents did not differ significantly between the four groups (ANOVA, $F_{1,85}$: 0.268, P= 0.606). The same result was found for the variation of standard length ($F_{1,85}$: 0.648, P= 0.423).

The time one individual spent chasing the other one within the pair was influenced by the pre-exposure of the audience ($F_{1,85}$: 7.388, P= 0.008) (**Figure 3.2**), with pairs pre-exposed to the audience spending more time in chasing (309.00 ± 36.79 s, N =42) than pairs not exposed to it (186.62 ±27.80 s, N=47). However, pre-exposure to an audience or exposure to it during the aggressive contest did not affect significantly any other measured behaviours. We found that resolved interactions between males that occurred in the presence of an audience, had a lower escalation index value (0.69± 0.049, N=46) than contests without an audience (0.84 ± 0.046, N=43)($F_{1,61}$: 4. 2156, P=0.044) (**Figure 3.3**).

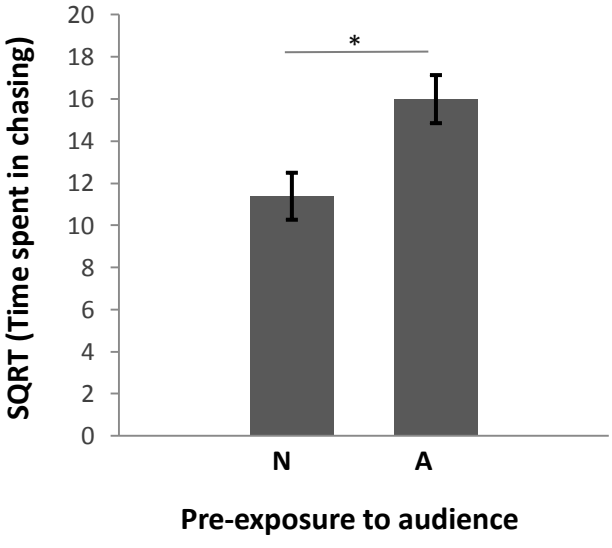

Figure 3.2. **The influence of pre-exposure to an audience in the time spent chasing (transformed variable)**. N – No audience; A – Audience Present.

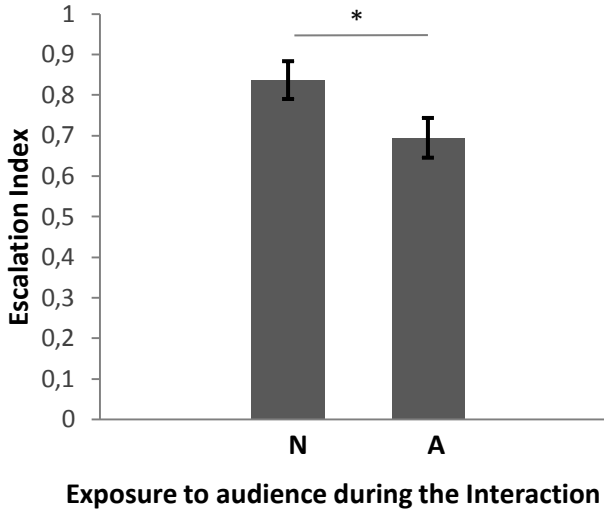

Figure 3.3. The influence of the exposure to an audience in the value of the Escalation Index of resolved fights. N – No audience; A – Audience present.

The percentage of interactions with assessment in the AA group significantly differed from all other groups (Pearson's chi-square test. For AA vs. AN: $X^2_1 = 12.600$, $P = 0.0004$; for AA vs. NA: $X^2_1 = 14.882$, P = 0.0001; For AA vs. NN: $X^2_1 = 10.761$, $P = 0.001$), while no differences were found between the AN, NA and NN groups (**Figure 3.4**).

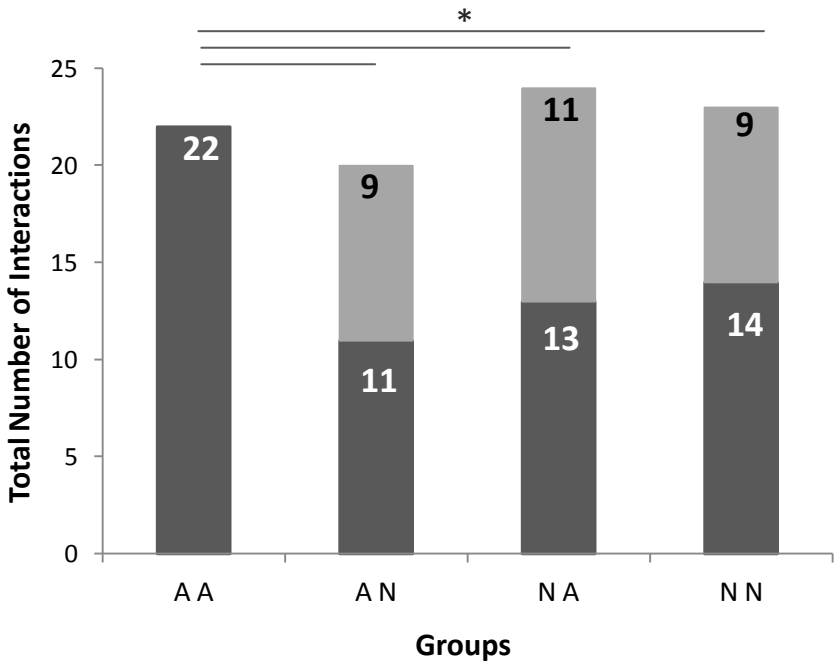

Figure 3.4. Proportion of interactions with and without assessment. Dark grey - Number of interactions with assessment; Light grey - Number of interactions without assessment. For the AA group, in 22 interactions (100%), assessment occurred between the two males. In the AN group, this proportion decreased to 11 out of 20 interactions (55%) while in the NA group, it decreased to 13 out of 24 (54,17%). In the NN group, the proportion of interactions with initiation of assessment was 14 out of 23 interactions (60,87%). Significant differences between groups are marked.

Neither the presence of an audience before or during the interactions influenced the occurrence of interaction, that is, a clear establishment of dominance (**Figure 3.5**). However the presence of an audience during the interactions influenced the percentage of resolved contests in which an assessment phase had occurred (two-tailed Fisher's Exact test: *P*=0.001)(**Figure 3.5**). When multiple comparisons were performed, we found that pairs in the AA group had more post-assessment resolutions than AN (two-tailed Fisher's Exact test: *P* =0.00006), NA (two-tailed Fisher's Exact test: *P* = 0.003) and NN groups (two-tailed Fisher's Exact test: *P* =0.0002).

All other comparisons were non-significant (two tailed Fisher's Exact test: AN vs. NA: *P*=0.327 ; AN vs. NN: *P*= 1.000; NA vs. NN: *P*=0.473).

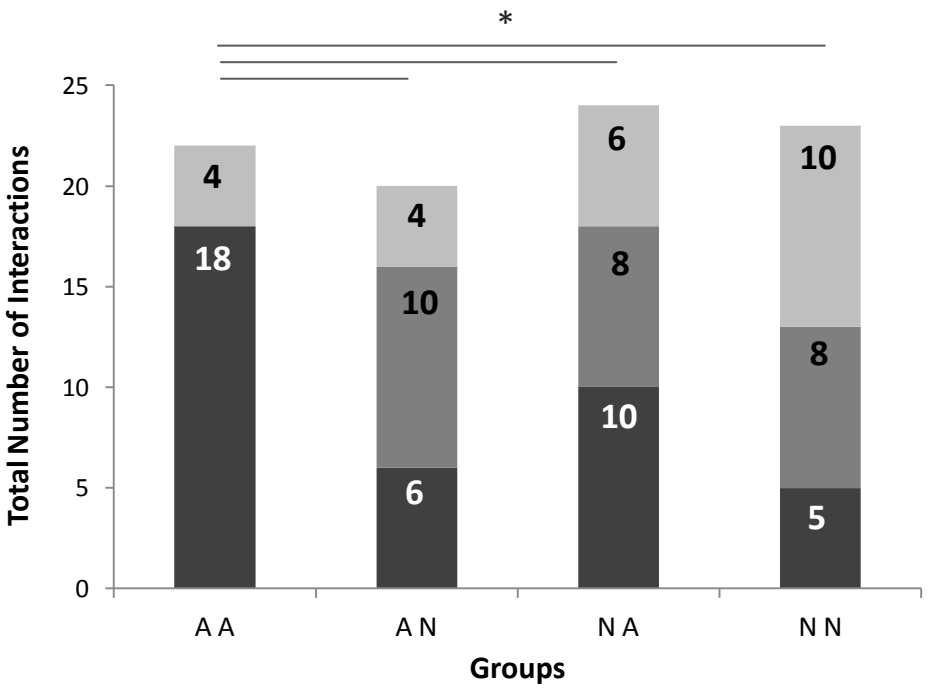

Figure 3.5. Proportion of resolved interactions, with our without assessment. Dark grey – Resolved interactions with assessment; Medium Grey – Resolved interactions without assessment; Light Grey – Unresolved interactions. In the AA group, out of 18 interactions, all occurred after assessment (100%) while in the AN group, out of 16 resolved interactions, 6 occurred after assessment (37,5%). In the NA group, this occurred in 10 out of 18 resolved interactions (55,56%) and in the NN group, this is true for 5 out of 13 resolved interactions (38,46%). Significant differences are marked

DISCUSSION

In this experiment, we aimed to understand if the presence of conspecifics affect aggressive signaling behaviour of observed males, in zebrafish. Our results demonstrate that zebrafish males alter their agonistic signaling behaviour in the presence of conspecific bystanders. Indeed, the presence of a mixed-sex audience during aggressive interactions promoted a decrease in overt aggression, while an assessment phase in the interactions was more likely to occur.

This mutual assessment usually takes place before a clear dominance relation between males is established, being characterized by the occurrence of displaying behaviour (Oliveira et al., 2011). However, as our results confirm, dominance can be established without it, which indicates that males exposed to conspecifics are, initially, more motivated to dispute dominance status by indirect, rather than by direct aggressive behaviour, such as biting.

Using indirect aggressive behaviours, such as displays, reduces the obvious costs and risks associated with engaging in direct aggression. It is also known that in zebrafish the cost of losing an aggressive interaction is higher than the benefit of winning it, as indicated by loser effects being more pronounced than winner effects in this species (Oliveira et al., 2011). If such effects were found in interactions that occurred without the presence of other individuals, one might expect that the costs of losing an interaction could be amplified in the presence of an audience. That is, losing an aggressive interaction can be more adverse when the individual is being observed (Peake & Mcgregor, 2004).

Several studies have reported that bystanding individuals can eavesdrop on others' agonistic interactions (Amy & Leboucher, 2009; Earley & Dugatkin, 2002; McGregor, Peake, & Lampe, 2001; Oliveira et al., 1998; Peake et al., 2001) and use the gathered information to assess others' fighting abilities (McGregor, 1993), therefore potentially affecting the observed males' fitness. Actually, in a study made with *Astotilapia burtoni* (Grosenick et al., 2007), males inferred the dominance status of other males after observing a series of agonistic interactions between them, indicating that also fish are

able of transitive inference. This reinforces our perception that losing in front of conspecifics might come at a higher price.

Therefore, we hypothesized that a loser which invested more energy into direct aggression towards its opponent might pay a higher cost when other individuals are present. In other words, investing high levels of energy in an agonistic encounter associated with the higher costs paid by losing in front of an audience might outdo the benefits of winning. As a result of this, males would be more likely to invest in low-cost aggressive behaviours, when an audience is around. This would, then, result in interactions with a lower escalation index when compared with interactions in which no access to the audience was allowed.

In the AA group, this seems to be more pronounced. Indeed, when compared to the other groups, the number of interactions with assessment, as well as the number of resolutions through assessment, is significantly higher. This might indicate that a prolonged exposure to conspecifics had a higher impact on the manipulation of males' signaling behaviour.

This group is the one that best represents the zebrafish natural environment since individuals are constantly exposed to other males and females (Spence et al., 2008). We know that zebrafish males defend oviposition sites from other rivals that swim nearby (Spence & Smith, 2005) and that they can establish these defensible territories in a matter of a few hours (Pérez-Escudero et al., 2014). In this scenario, the decrease in overt aggression towards a rival seems more likely. Displaying towards rivals might be a low-cost and effective way to prevent them from entering their territory without the need to engage in direct aggression with each rival. In the AA group, both males spent 24 hours facing a group of conspecifics which included two potential rivals. When a new and more emergent treat came by, males might have addressed it by resorting to low-cost displays as a way of defending they territory since more potential treats were nearby.

Our results also indicate that pre-exposure to an audience influenced chasing behaviour after the establishment of dominance. That is, winners increased the time spent actively

chasing the losers, therefore reinforcing their dominance status. This is consistent with the emergence of aggressive priming (Hogan & Bols, 1980) in zebrafish. In this case, pre-exposure to conspecifics lead to an increase of aggressive behaviour. Curiously, this effect was only detectable after the dominance status was established while, in other species, it mainly affects the latency until the first attack (Hogan & Bols, 1980; Matos et al., 2003; Potegal & Popken, 1985). For instance, in the Siamese fighting fish, latency to bite decreased in males pre-exposed to other males (Matos et al., 2003).

All combined, our results suggest that audiences affect agonistic signaling behaviour of zebrafish males. This reinforces the body of work produced so far which indicates that the audience effect is a widespread phenomenon (Bertucci et al., 2013; Blum et al., 2008; Claire Doutrelant, Mcgregor, & Oliveira, 2001; T. Dzieweczynski, Gill, & Perazio, 2012; T. L. Dzieweczynski et al., 2014; le Roux et al., 2008; Leaver et al., 2007; Marler et al., 1986; Milner et al., 2012; Ziege et al., 2009). However, it has mainly been studied from a behavioural or hormonal (Dzieweczynski, Eklund, & Rowland, 2006) perspective. Here we validated the use of this behavioural paradigm to study audience effects in a model organism with more available genetic tools (Oliveira, 2013) than other previously studied species. This will allow, in future work, to deeply explore the neural circuits and gene networks underlying the use of social information.

4. Final Remarks

Here, we wanted to determine if two phenomena associated with the existence of communication networks, eavesdropping and audience effects, occur in zebrafish, in an agonistic context.

We were not able to detect the occurrence of eavesdropping although we could determine that zebrafish males adjust their signaling behaviour when conspecifics are present before and/or during an aggressive interaction. The fact that we could not demonstrate the occurrence of eavesdropping in this species does not mean that it does not occur. Indeed, the existence of an audience effect in zebrafish males suggests that eavesdropping occur. Therefore, a new paradigm to study eavesdropping needs to be established, considering the suggestions made before.

These two paradigms will allow a better understanding of communication networks in social species and behavioural plasticity, in this case, how animals use others' signals to their benefit and how they adjust their behaviour according to the presence of conspecifics.

Studying these phenomena in zebrafish can lead, in the future, to a more complex approach since other studies have been mainly focused on the behavioural or hormonal perspectives (Dzieweczynski, Eklund, & Rowland, 2006). Indeed, as stated before, zebrafish is a model organism with a high number of genetic tools and transgenic or mutant lines available (Stewart et al., 2014). These resources can be used in futures studies to better understand the gene networks and neural circuits underlying both these phenomena. For instance, we could use genetic markers of neural activity (i.e. immediate early genes) (Taborsky & Oliveira, 2012) to map the activation of brain areas involved in the use of socially acquired visual information and to determine the effect of the presence of conspecifics in the modulation of aggressive behaviour in males, also at a neural network level. The use of mutant or transgenic lines can also be valuable, since it could allow to selective activation or knockout of brain areas involved in these two phenomena.

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6. Supplementary Information

6.1. EAVESDROPPING EXPERIMENTAL PROTOCOL

Day 1

Manipulation of the bystanders' "dominant" or "subordinate" status

1. Remove 4 fish (2 will be the experimental subjects and the other 2 will be the control) from the general stock. Place each fish in one half of two 15x15 cm arenas divided by an opaque partition, with a 9 cm water height;

2.Place a LED light above the arenas and feed the fish with *Artemia Salina*;

3. Leave the fish visually separated for 24 hours but in chemical contact with each other.

Day 2

4. Remove the opaque partitions that separate the fish in both arenas;

5. Let the fish interact for 30 minutes. Record the interactions with a video camera in order to detect the winner and loser. During this period of time leave the room to minimize external influences that could prevent the dominance establishment from occurring.

6. After 30 minutes, separate the individuals with the same opaque partitions used before;

7. Watch the recorded video to determine which individuals won and lost the interaction. The winners will become the dominant bystanders (D) while the losers will be the subordinate bystanders (S);

8. Place each fish (Experimental and Control Fish) in the corresponding bystander arenas of the experimental set up. The order by which dominant and subordinate bystanders are placed is randomized. Feed all fish with *Artemia salina*.

Habituation to demonstrator fighting fish

9. From the demonstrators stock, remove six fish. These will be placed in tanks T1 to T4 (30 x 15 cm), on the nearest half to the bystanders' arenas, with a 9 cm water height and separated with opaque partitions in the experimental set up;

10. Place a LED light above the tanks, in order to create a one-way mirror effect. Bystanders will have visual contact with the demonstrator in order to habituate.

11. Leave the fish overnight.

Day 2

Social Learning Test

12. Set up the electronic equipment used to record and track behavior from the experiment (cameras, computer, cables etc.) and follow protocol timeline (**Fig1**);

13. For tanks T1 and T2 record the corresponding bystanders' behavior during 30 minutes (**BL Timepoint**) with top-view cameras in the bystander tanks;

14. Next, If T1 and T2 randomization process corresponds to experimental bystanders (ED and ES), place opaque partitions between bystander and tanks T1 and T2, which prevents the observation of the demonstrators, during 30 minutes (**BO Timepoint**). At the end of this period of time, remove again the opaque partitions for the experimental bystanders tanks. If A and B corresponds to control bystanders (CD and CS), don't place the opaque partition during these 30 minutes.

15. Record once more bystanders' behavior during 30 minutes with the top-view cameras (**BI Timepoint**);

17. Remove the partitions that separate the demonstrator fighters and let them interact (if T1 and T2 tanks corresponds to control bystanders place an opaque partition between them and demonstrator fighting fish). Record both interactions with a second synchronized front camera, for 30 minutes, in order to determinate the winner (W) and

loser (L) (**Figure1**). Also, record the bystanders' behavior (control and experimental) during these contests with the top-view cameras (**IO / IN Timepoint**);

18. After this period of time, separate the demonstrators with a partition. (If the case, remove the opaque partition which prevented control bystanders from observing the fight);

19. For the next 30 minutes, record again both bystanders' behavior with the top cameras (**AI Timepoint**). Record as well the demonstrator fish during this period of time, with the front camera.

20. Repeat steps 13 to 19 for tanks T3 and T4;

21. Wash the experimental set up with water and alcohol and prepare it for next session;

22. Track and analyze the behavioral recordings.

NOTE1: Use a flash light in order to synchronize top cameras and fighting camera recordings. Use it after the cameras start recording.

NOTE2: Always leave the room when recording behaviors.

6.2. AUDIENCE EFFECTS EXPERIMENTAL PROTOCOL

Day 1

Habituation Period

1. Remove 8 fish from the subjects' stock. Place each fish in one half of the 15 x 15 cm fighting arenas, divided by an opaque partition, with a 9 cm water height.

2. If subjects will be submitted to treatments AA or AN on the following session, remove the opaque partitions between the fighting arenas and the audience tank. Otherwise, maintain them.

3. Feed the subjects with live food (*Artemia salina*) and the audience elements, with both live food and crushed TetraMin tropical fish food flakes.

4. Slide the black curtains and leave the subjects to habituate for 24 hours.

Day2

Interaction Period

5. If the Fighting Arena1 randomization process corresponds to:

1) Treatment AA - Place and remove one opaque partition between the fighting arena and the audience tank.

2) Treatment AN - Place one opaque partition between the arena and the audience tank.

3) Treatment NA - Remove the opaque partition placed between the arena and the audience tank.

4) Treatment NN - Remove and place once more the second opaque partition, between the fighting arena and the audience tank, maintaining the other one in place.

6. Remove the opaque partitions separating the subjects in the fighting arena and let them interact for a 30 minutes period. Record the interaction with a video camera, through the protective barrier's hole.

7. After this period of time, separate the subjects with an opaque partition.

8. Repeat steps 5 to 7 for the Fighting Arenas2, 3 and 4, in this exact same order.

9. Wash the Fighting arenas with water and alcohol and prepare them for the next session.

NOTE1: At the end of each week use a sponge to clean accumulated algae in the audiences' tanks. Change one third of the water and replace it by clean maturated water.

NOTE2: Always leave the room when recording behaviors.